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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 38/21, 45/00, C12N 5/08, C07K 14/52, 14/55, 14/705, 16/28

A1

(11) International Publication Number: **WO 95/32729**

(43) International Publication Date: 7 December 1995 (07.12.95)

(21) International Application Number: PCT/US95/06703

(22) International Filing Date: 31 May 1995 (31.05.95)

(30) Priority Data:
08/251,860 31 May 1994 (31.05.94) US

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: SHORT-TERM ANTI-CD3 STIMULATION OF LYMPHOCYTES TO INCREASE THEIR *IN VIVO* ACTIVITY

(57) Abstract

A tumor-bearing mammal is treated by stimulating lymphocytes, including T lymphocyte subsets, *in vitro* in the presence of an antibody to a lymphocyte surface receptor for less than about 24 hours, optionally in the presence of IL-2, to form stimulated lymphocytes; infusing the stimulated lymphocytes into the tumor-bearing mammal; and administering one or more lymphokines to the mammal. The stimulated lymphocytes display enhanced immunotherapeutic activity, including increased cytotoxicity or lymphokine production *in vivo*, resulting in a decrease in the tumor burden by at least about 20 %.

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SHORT-TERM ANTI-CD3 STIMULATION OF LYMPHOCYTES TO INCREASE THEIR *IN VIVO* ACTIVITY

Field of the Invention

The present invention relates to the culturing of lymphocytes in the presence of a lymphocyte surface receptor antibody so as to elicit an immune response *in vivo*. Specifically, it relates to the short-term stimulation of lymphocytes *in vitro* with anti-CD3 monoclonal antibody followed by the reintroduction of the activated lymphocytes *in vivo*. The lymphocytes may comprise a total lymphocyte population, or alternatively, a depleted or positively selected lymphocyte population. The invention is particularly represented by improved antitumor activity.

Background of the Invention

T lymphocytes, i.e., T cells, are central players in the immune response by virtue of their ability to recognize antigens with a high degree of specificity, to act as effector cells in the lysis of specific target cells, and to regulate the nature and intensity of the immune response. Once research clarified the role of T lymphocytes in the immune system, it became useful to stimulate selected lymphocyte populations and subpopulations that could play a role in immune responses, especially in response to tumors.

Interleukin-2 (IL-2), a lymphokine produced by helper T cells, stimulates the growth of T cells and NK cells that bear IL-2 receptors, either *in vivo* or *in vitro*. The *in vitro* incubation of resting lymphocytes in media containing IL-2 for three to four days induces the generation of lymphocytes capable of lysing fresh tumor cells, but not normal cells. These lymphocytes are

referred to as lymphokine activated killer (LAK) cells. See, for example, I. Yron et al., J. Immunol., 125, 238 (1980); M.T. Lotze et al., Cancer Res., 41, 4420 (1981); and S.A. Rosenberg et al., Natl. Cancer Inst., 75, 595 (1985). This ability of IL-2 to induce the proliferation of lymphocytes with immune reactivity and with the ability to lyse fresh autologous, syngeneic, or allogeneic natural killer (NK) cell resistant tumor cells, but not normal cells, has resulted in further developments in the area of adoptive immunotherapy, i.e., cell transfer therapies.

Typical adoptive immunotherapy involves the administration of immunologically active cells to an individual for the purpose of providing a beneficial immunological effect to the individual, e.g., reduction or control of cancerous or diseased tissue. These immunologically active cells, e.g., lymphokine-activated killer cells and tumor infiltrating cells, are typically taken either from the individual to be treated, known as an autologous treatment, or from another individual, known as an allogeneic treatment. Lymphocytes are typically taken by venipuncture or leucophaeresis. Tumor infiltrating cells are taken from tumors removed during surgery. The lymphocytes are cultured to increase their number and activate their antitumor activity, and infused back into the patient. Thus, the majority of conventional efforts in adoptive immunotherapy are directed at increasing the number of activated cells *in vitro* followed by infusion back into the patient.

Animal experiments transferring immunologically active cells, e.g., LAK cells, from healthy animals to animals with cancerous tumors have suggested that adoptive immunotherapy can elicit an antitumor effect in certain tumor models with a high degree of effectiveness. Furthermore, the administration of IL-2 in addition to LAK cells has proven effective in the treatment of a variety of murine malignancies. IL-2 also leads to the *in vivo* proliferation of transferred LAK cells. These

initial animal studies were repeated with humans in clinical trials.

The human studies demonstrated that LAK cells plus IL-2, or IL-2 alone, can be effective in mediating the regression of established metastatic cancer in selected patients. See, for example, S.A. Rosenberg, "Immunotherapy of Patients with Advanced Cancer Using Interleukin-2 Alone or in Combination With Lymphokine Activated Killer Cells" in Important Advances in Oncology 1988, J.B. Lippincott Co., 217, (1988).

Although adoptive immunotherapy has met with certain success, a difficulty with many of the experimental protocols is that a large number of cells is required in the therapy. Furthermore, conventional protocols have proven less than desirable because of the large amount of culture medium, the large amount of IL-2, the large number of hours involved in culturing cells to develop LAK activity, the time involved in clinical treatment, and the side effects of treatment. The infusion of LAK cells also frequently results in the development of respiratory difficulty. These cells are large and sticky and bind to the vascular system in the lungs causing severe damage that can result in the patient requiring intensive care management.

To overcome some of these difficulties, advances have been made to improve the *in vitro* culturing process. For example, T cells cultured in the presence of IL-2 and monoclonal antibodies (MoAb) against the antigen receptor complex CD3, i.e., anti-CD3 MoAb, have been found to proliferate and demonstrate *in vitro* LAK activity on a per cell basis. See, for example, P.M. Anderson et al., Cancer Immunol. Immunother., 27, 82 (1988); P.M. Anderson et al., J. Immunol., 142, 1383 (1989); and A.C. Ochoa et al., Cancer Res., 49, 963 (1989).

Some efforts have been aimed at activating *in vivo* antitumor mechanisms, however, there has been limited success in this area. For example, patients have received high doses of IL-2 with significant toxicity.

The direct infusion of anti-CD3 monoclonal antibody alone induces nonspecific antitumor function in mice. See, for example, D.W. Hoskin et al., Cancer Immunol. Immunother., 29, 226 (1989). Based on the positive results in murine models, direct infusion of anti-CD3 MoAb has been attempted in humans. Although patients who have directly received the anti-CD3 MoAb OKT3 have experienced the activation of some T cells *in vivo*, the toxicity of intravenous OKT3 reaches the maximum tolerated dose (MTD) before immune efficacy develops. It is believed that the free OKT3 is responsible for the majority of these toxic effects. In addition, the infusion of anti-CD3 induces the production of endogenous antibodies (human anti-mouse antibodies or HAMA) which neutralize the effect of the anti-CD3 thus complicating the possibility for repeated therapy.

Although direct infusion of anti-CD3 results in significant levels of toxicity, cells activated by anti-CD3 outside of the body are useful in adoptive immunotherapeutic techniques. Anti-CD3 induces, at least in part, activation of tumoricidal T cells as well as activated NK (LAK cells). Conventional IL-2 therapy, however, only activates LAK cells. Furthermore, some studies have shown that certain tumor target cells are more susceptible to anti-CD3 activated T cells than LAK cells. See, for example, J. Stankova et al., Cell. Immunol., 121, 13 (1989) and Yun et al., Cancer Res. 49, 4770 (1989).

A comparison of the efficacy of cells stimulated with IL-2 for four days, with anti-CD3 for 72 hours, as well as the antitumor activity induced by the direct infusion of anti-CD3 MoAb has been undertaken. See, for example, S. Gallinger et al., Cancer Res., 50, 2476 (1990). It has been determined that in this murine model the most effective way of reducing tumor is direct infusion of anti-CD3. Infusion of cells stimulated with anti-CD3 was less effective while infusion of cells cultured in IL-2 was least effective.

It has been shown that anti-CD3 induced effector cells acquire cytolytic activity *in vitro* within 24 hours of incubation of murine lymphocytes with anti-CD3. It has also been shown that *in vitro* incubation of peripheral blood lymphocytes of normal donors with the anti-CD3 monoclonal antibody OKT3 for 45 minutes resulted in the acquisition of lytic activity against fresh leukemic cells. See, E. Lotzova et al., Nat. Immun. Cell Growth Regul., 6, 219 (1987). It was generally believed, however, that for effective *in vivo* activity, cells must be incubated with anti-CD3 for at least about 72 hours.

Thus, conventional methods of activating cells *in vitro* or *in vivo* have not to date successfully eliminated the need for convenient, effective, and safe therapeutic methods. In general, conventional methods require the development of immunotherapeutic function, e.g., lytic activity, in cells before they are used in adoptive immunotherapy. Because of this limitation, culturing of cells can last for several days before they can be infused back into a patient. A need therefore exists for a more convenient, less time consuming, and more efficient method of culturing cells. That is, a need exists for a method of producing lymphocytes capable of proliferation and enhanced immunotherapeutic efficacy that: (1) is not time consuming; (2) is less expensive; (3) has fewer, if any, side effects; (4) is simple and convenient; (5) does not require prolonged *in vitro* culture; and (6) can activate any and all types of T cells required for immunotherapy.

Patients subjected to a bone marrow transplantation encounter a period of severe immune deficiency following high dose chemotherapy and/or total body irradiation (TBI). The patient's bone marrow is replaced with a small amount of healthy bone marrow cells that proliferate in the body until enough marrow cells have been generated to achieve a repopulation of the peripheral blood by red blood cells, platelets and white blood cells of the immune system. A typical bone marrow

engraftment can take anywhere from 20 to 30 days to regenerate (engraft) sufficient quantities of bone marrow cells. During this period, the patient's immune system is virtually non-functional, and the patient must be closely monitored to prevent the onslaught of disease. These bone marrow cells can be induced to proliferate with factors such as G-GSF, GM-CSF, IL-3 and the like. However, all of these factors have to be administered at high doses that may be toxic to the patient. Thus, there exists a need to provide a method that is capable of stimulating the proliferation of bone marrow cells to increase the number of bone marrow cells produced and thereby decrease the time needed to regenerate a sufficient quantity of bone marrow cells. There also exists a need to provide a method that is capable of eliminating residual tumor cells in the bone marrow.

Summary of the Invention

It is an object of the present invention to provide a method of enhancing the *in vivo* immune response, i.e., the immunotherapeutic activity, of lymphocytes. It also is an object of the invention to provide a method of treating a mammal having tumors using an immune cell population having an enhanced immunotherapeutic activity against these tumors. It is an additional object of the present invention to provide a method for reducing the tumor burden in mammals.

In accordance with these and other objectives of the present invention, there is provided a method of enhancing the immunotherapeutic activity of lymphocytes by stimulating the lymphocytes *in vitro* in the presence of an antibody to a lymphocyte surface receptor, optionally in the presence of a low level of IL-2, for less than about 24 hours to form stimulated lymphocytes, infusing the stimulated lymphocytes into a tumor-bearing mammal, and administering an effective amount of IL-2 to the mammal. The antibody to a lymphocyte surface receptor is preferably anti-CD3.

The lymphocytes may comprise a total lymphocyte population, or alternatively, a depleted or positively selected lymphocyte population. By separating at least one mutually inhibiting cell subset within an immune cell population, the remaining depleted immune cell population or the separated cell subset is capable of more fully expressing its immune function when stimulated. The depletion method for preparing a depleted lymphocyte population includes separating at least one cell subset, or subpopulation, that is capable of down-regulating the immunotherapeutic activity of an immune cell population, from that immune cell population to form a depleted immune cell population; and culturing the depleted immune cell population in the presence of an antibody to a lymphocyte surface receptor, optionally in the presence of IL-2, to form a stimulated depleted immune cell population. Preferably, this method reduces or eliminates a regulatory mechanism from the immune cell population which allows the remaining cells to more fully express their immune function. The immunotherapeutic activity of the remaining cell population, as represented by a measure of the ability of the immune cell population to reduce tumor volume, is increased when compared to a similarly treated undepleted immune cell population.

The positive selection method of enhancing the immunotherapeutic activity in accordance with an object of the present invention includes separating and positively selecting at least one cell subset, or subpopulation, that is capable of up-regulating the immunotherapeutic activity, or developing the immunotherapeutic activity, of an immune cell population, from that immune cell population to form an immune cell subset; and then culturing the immune cell subset, e.g. $CD4^+$ or $CD8^+$ cells, in the presence of an antibody to a lymphocyte surface receptor, to form a stimulated immune cell subset. The immunotherapeutic activity of the positively selected and stimulated immune cell subset, as represented by a measure of the ability of the immune

cell subset to reduce tumor volume, is increased when compared to a similarly treated total lymphocyte population.

In accordance with the methods described above, the total lymphocyte population, or alternatively, a depleted or positively selected lymphocyte population, is cultured in a medium in the presence of an antibody to a lymphocyte surface receptor, optionally in the presence of IL-2, for less than about 24 hours, e.g., for 12 to 18 hours, more preferably less than 4 hours, and as short as 30 minutes. Preferably, the antibody to a lymphocyte surface receptor is anti-CD3. If IL-2 is present in the culture medium, it is present in low doses (<300 units/ml), in contrast to high doses and long exposure as was practiced in the prior art. If IL-2 is present in the culture medium during stimulation of lymphocytes *in vitro*, it is an amount of IL-2 that is sufficient to maintain the viability of cells without inducing substantial lymphocyte proliferation.

In accordance with the methods described above, the total lymphocyte population, or alternatively, a depleted or positively selected lymphocyte population, can be administered to the patient after stimulation with the antibody against the surface receptor. These cells will then proliferate *in vivo* after IL-2 is administered, preferably liposomal IL-2. While not intending to be bound by any theory, it is believed that culturing the immune cell population, or alternatively, a depleted or positively selected lymphocyte population, in the presence of an antibody to a lymphokine surface receptor "primes" the cells to upregulate the production of IL-2 receptor sites on the cells. Thus, when IL-2 is further administered either *in vivo* in conjunction with the stimulated cells, or *in vitro*, the stimulated cells bind more IL-2 and a higher level of cytotoxic activity is induced. These stimulated cells, when bound with IL-2, then are capable of lysing tumors because the IL-2 may

generate a signal cascade within the cells that results in LAK activity.

Immune cell populations stimulated in accordance with any of the foregoing methods are useful for the following methods of treatment. In accordance with an additional object of the present invention there is provided a method of treating a mammal having tumors with a stimulated total lymphocyte population, or alternatively, a depleted or positively selected lymphocyte population having an enhanced immunotherapeutic activity, prepared by the procedures outlined above. This method therefore includes (i) enhancing the immunotherapeutic activity of an immune cell population as described immediately above; (ii) administering the immune cell population to a mammal having tumors and optionally pretreated with an immunosuppressant; and (iii) administering IL-2, preferably liposomal IL-2, to the mammal in addition to the immune cell population of step (ii). As a result of these methods, the anti-CD3 stimulated lymphocytes display enhanced immunotherapeutic efficacy *in vivo* as represented by a decrease in the tumor burden by at least about 20%. The short-term stimulation of the lymphocytes can be effected over a period of about 12-18 hours, more preferably less than 4 hours, and as short as 30 minutes, and the tumor burden is decreased by at least about 40%, more preferably by at least about 60%, and most preferably by at least about 80%.

In accordance with yet another object of the present invention, there is provided a method of stimulating the proliferation of bone marrow cells comprising the steps of first enhancing the immunotherapeutic activity of an immune cell population in accordance with any of the procedures outlined above. The stimulated cells are then incubated in the presence of bone marrow, optionally in the presence of additional cytokines including granulocyte macrophage colony stimulating factor (GM-CSF), IL-3, Kit Ligand (KL), erythropoietin (EPO) or IL-2 to thereby increase the number of bone marrow cells. The

bone marrow cells, cultured *in vitro*, then can be administered to a mammal to stimulate the proliferation of additional bone marrow cells. Alternatively the stimulated lymphocytes can be administered to a mammal having a compromised bone marrow cell population to stimulate growth of bone marrow cells *in vivo*.

In accordance with yet another object of the present invention there is provided a method of enhancing the engraftment of bone marrow cells and/or killing residual tumor cells from bone marrow cells comprising the steps of first enhancing the immunotherapeutic activity of an immune cell population in accordance with any of the procedures outlined above. The stimulated cells are then infused, optionally together with bone marrow cells, into the patient. The patient is optionally treated with a lymphokine(s) such as IL-2.

Brief Description of the Drawings

Fig. 1 shows the comparative increase of IL-2 receptors after 24 hours of stimulation in varying concentrations of anti-CD3.

Fig. 2 shows the increase in the expression of the α chain of the IL-2 receptor on human T lymphocytes after overnight incubation with anti-CD3.

Fig. 3 shows human lymphocyte growth after 24 hour stimulation with anti-CD3 and subsequent culturing in media containing IL-2.

Fig. 4 shows the growth of lymphocytes stimulated with differing concentrations of anti-CD3 for four hours followed by culture in IL-2 containing medium.

Fig. 5 shows the white blood cell count from a patient whose lymphocytes were cultured overnight in anti-CD3. The lymphocytes were infused back into the patient and the patient subsequently received IL-2 treatments.

Detailed Description

As used herein, "stimulating" or "culturing" indicates the process whereby cells are placed in a tissue culture medium comprising nutrients to sustain the

life of the cells, and other additives, such as the anti-CD3 monoclonal antibody. Stimulation can be done in the presence of IL-2 but generally IL-2 is not included. If stimulation of lymphocytes is to be done in the presence of IL-2, low doses of IL-2 (<300 units/ml) are used in contrast to the high and long exposure as was practiced in the prior art. This process can take place in any vessel or apparatus. The process can involve various stages of culturing and subculturing. However, typically, only one culturing or stimulating step is desirable. IL-2 activity is expressed herein in Hoffmann-LaRoche units. 2.3 Hoffmann-LaRoche units equal 6 International Units.

As used herein, the phrase "depletion method" denotes a method of enhancing the immunotherapeutic activity of a total lymphocyte population by utilizing a "depleted lymphocyte population." A depleted lymphocyte population is the portion of the total lymphocyte population remaining after at least one cell subset, or subpopulation, that is capable of down-regulating the immunotherapeutic activity of that lymphocyte population, has been separated and removed therefrom. The resulting depleted lymphocyte population, when stimulated, is referred to herein as a "stimulated depleted lymphocyte population."

As used herein, "depleted lymphocyte population" preferably refers to a total cell population derived from a sample of peripheral blood, spleen, lymph node, tumor, pleural effusions, *in vitro* cultured lymphocytes, and the like, with at least one cell subset or subpopulation, which down-regulates the immunotherapeutic activity of the total population, removed therefrom. A "depleted lymphocyte population" can be further depleted by removal of a subpopulation or subset, e.g., $CD4^+$ from which a subset, e.g., 2H4, is removed.

Throughout this description, the phrase "positive selection method" denotes a method of enhancing the immunotherapeutic activity of a lymphocyte population by

using an "lymphocyte subset." A lymphocyte subset is the portion of the lymphocyte population that has been separated and removed, or panned from the lymphocyte population. This lymphocyte subset, when stimulated, is referred to herein as a "stimulated lymphocyte subset."

As used herein, "immune response" or "immunotherapeutic activity" refers to any of a variety of immune responses of immune cells. This includes antitumor activity, such as lymphokine production, specific lytic activity, or the nonspecific lytic activity of lymphokine activated killer (LAK) cells and natural killer (NK) cells. As used herein, LAK activity is defined as the ability of lymphocytes to lyse tumor cells, and to a lesser degree normal cells. This activity in lymphocytes is typically stimulated by lymphokines, such as IL-2. NK activity is defined as the ability to lyse tumor cells, but not normal cells, which does not result from prior stimulation. Furthermore, as used herein "antitumor activity" includes a reduction in the number of tumors as well as a reduction in the size of tumors which can be collectively referred to as reduction in tumor burden.

As used herein, "immunotherapeutic activity" also includes suppression of an undesirable immune response. T lymphocytes can also function as suppressor cells such that they inhibit an on-going immune response. It is therefore expected that some of the T lymphocyte subsets, activated according to the present invention, will be useful in treatment of diseases where the immune response produces damage to normal tissues, e.g., autoimmune disease.

The increased immunotherapeutic activity of lymphocyte populations can be determined *in vitro* by the percent cytotoxicity, which is a measure of the ability of immune cells to destroy a radioactively labeled tumor target. That is, the antitumor activity is determined by a comparison of the level of radioactivity released in tissue culture media from the effector/target combination

to the level of radioactivity in the culture media released from the target alone. Thus, increased immunotherapeutic activity, as defined herein, of immune cells is typically demonstrated by an increase in the percent cytotoxicity of the effector cells on tumor cells. The tumor cell lines can be any of a variety of cell lines commercially available, including leukemia cells and fresh tumor targets. Preferably, they are leukemia cells. For example, cytolytic activity can be measured quantitatively using the cell-mediated lympholysis assay (CML) as described in S.-L. Wee et al., Hum. Immunol., 3, 45 (1981), which is incorporated herein by reference.

As used herein "lymphocytes" include T cells, such as $CD3^+$ T cells, including $CD4^+$ and $CD8^+$ cells. It is understood that this includes tumor infiltrating lymphocytes (TIL cells). Preferably, the lymphocytes are T cells, or T cell subsets, including, but not limited to $CD4^+$, or any $CD4^+$ subsets such as 2H4 or 4B4, $CD8^+$, or any of its subsets.

It has been discovered that lymphocytes can be stimulated *in vitro* with an antibody to a lymphocyte surface receptor, without the presence of IL-2, for short periods of time, i.e., for less than about 24 hours, e.g., 12-18 hours and as short as 30 minutes. These cells have a high therapeutic efficacy upon *in vivo* injection. Anti-CD3 stimulated cells are less toxic and are generally smaller in size than cells cultured only in IL-2 for several days. Reduced pulmonary toxicity may be due to the smaller size of anti-CD3 stimulated cells which presumably circulate more freely through the vascular system than larger LAK cells. Similarly, because the anti-CD3 stimulated cells can multiply in number in the presence of IL-2, small numbers of injected anti-CD3 stimulated cells can proliferate to large numbers following *in vivo* exposure to IL-2 allowing for the administration of fewer cells.

The antibody to a lymphocyte surface receptor can be any of a variety of monoclonal antibodies (MoAb) against a surface antigen receptor complex having mitogenic potential. For example, it can be an anti-CD3, anti-CD2, anti-CD4, anti-CD5, anti-CD28, anti-CD11b, etc., MoAb. It is preferably an anti-CD3 MoAb. The antibodies can be used alone or in various combinations with other antibodies. For example, anti-CD3 can be used in combination with anti-CD2, anti-CD4, anti-CD5, anti-CD28 or anti-CD11b, for effective results. The anti-CD3 MoAb used to stimulate human T cells can be, but is not limited to, OKT3, WT32, Leu-4, SPV-T3c, RIV9, 64.1, etc. The anti-CD3 MoAb used to stimulate murine T cells is more preferably the anti-murine CD3 MoAb 145-2C11, which has been identified by O. Leo et al., Proc. Natl. Acad. Sci. USA, 84, 1374-1378 (1978), and is available from American Type Culture Collection (ATCC). Mouse anti-human OKT3 is available from the Ortho Division of Johnson and Johnson.

Cells treated with anti-CD3 MoAb for less than about 24 hours are preferably treated with a total dose of about 0.1 ng/ml to 10 ng/ml anti-CD3 antibody, in stimulating one whole leucophaeresis. A "leucophaeresis" is defined as the peripheral blood lymphocytes (PBLs) enriched for mononuclear cells removed during the course of a standard phaeresis procedure. More preferably, anti-CD3 is used in a total dose of about 10 ng/ml used in stimulating one whole leucophaeresis.

The anti-CD3 stimulation induces the expression of the IL-2 receptor. These cells are then collected and placed in the body of an organism, preferably a mammal, such as a mouse or a human, where they develop immunotherapeutic activity, e.g., cytotoxic activity or lymphokine production, upon the administration of IL-2. More preferably the cells are placed in the body of a human for immunotherapeutic treatment.

Because cells stimulated for a short period of time with anti-CD3 *in vitro* are smaller than cells cultured

for 4 days in IL-2, or IL-2 in conjunction with anti-CD3, and because they have not had time to develop appreciable, if any, lytic activity, they are less toxic when administered *in vivo*. The administration to mice of large numbers (> 50 x 10^6) of cells stimulated with IL-2, or IL-2 and anti-CD3, and cultured for greater than 4 days, usually 5 to 6 days, can produce acute respiratory problems in mammals due to sludging, i.e., stasis or blocking the lung capillaries, by these large cells in pulmonary capillaries. In contrast, stimulation of cells with anti-CD3 alone or anti-CD3 + IL-2 for less than 24 hours does not generally produce an increase in the size of the cells. Furthermore, cells stimulated overnight with anti-CD3 proliferate, i.e., multiply, *in vivo* when administered to an animal receiving IL-2. Therefore, smaller numbers of cells can be administered.

With the administration of an effective amount of IL-2 *in vivo*, the cells display an enhanced proliferation and antitumor activity. The administration of IL-2 preferably occurs over a period of about 7 days. Lymphokines, e.g., IL-2, can be administered in free form or controlled release dosage form, e.g., liposomes. The amount of IL-2 effective for enhancing cell proliferation and antitumor activity *in vivo*, such that there is at least about a 20% decrease in the number of tumors, depends on the mammal being treated and also on the form in which it is administered. For example, the effective dose of IL-2 is expected to be lower when administered as liposomal IL-2 than when administered as free IL-2. About 10,000-70,000 units/day of IL-2, preferably about 50,000 units/day of IL-2, are administered to mice, and about 1 x 10^4 to 1.8 x 10^7 International Units/m^2/day are administered to humans. Certain stimulated depleted immune populations or stimulated lymphocyte subsets may exhibit enhanced proliferation and antitumor activity *in vivo* without having to administer lymphokines, such as IL-2, or with administration of only low doses of IL-2 to the patient.

As stated above, it is preferred that the cells are initially stimulated for less than 24 hours with anti-CD3 MoAb. While it is within the scope of the invention to include low doses of IL-2 with the anti-CD3 MoAb in the initial culture, if desired, it is more efficient to stimulate cells with anti-CD3 MoAb alone, collect the stimulated cells, infuse the stimulated cells into a tumor-bearing mammal, and then administer IL-2 to the mammal. As used herein, the phrase "relatively minor amount of IL-2" denotes a stabilizing amount of IL-2, *i.e.*, an amount sufficient to sustain the culture at about its initial cell density. Advantageously, IL-2 is present in an amount of less than about 30% of the amount of IL-2 typically used in culturing procedures. When lymphocytes are cultured optionally in the presence of IL-2, this typically will mean that if IL-2 is used at all, it is used in a "relatively minor amount." For example, if about 1,000 units of IL-2 typically are used in culturing a specific lymphocyte population, then less than about 300 units would be used in the present invention if culturing with IL-2 is optional, and IL-2 is utilized.

It is also within the scope of the invention to include one or more other lymphokines, in addition to, or in place of IL-2, in the initial culture with an antibody to the lymphocyte surface receptor. These include IL-1, IL-4, IL-6, IL-12, interferon, etc. It is envisioned that they can be used alone, in sequence, or in combination with low dose IL-2 as an adjunct to simulation with an antibody to a lymphocyte surface receptor.

It is also contemplated that one or more of these lymphokines can be administered to the patient following infusion of the lymphocytes stimulated with an antibody to a surface receptor. The lymphokines can be administered alone, in sequence, or in combination with IL-2. It is contemplated that administration of different lymphokines will play a role in selecting or

supplementing the immune response and effector mechanisms.

For example, the $CD4^+$ subset (TH) appear to control and modulate the development of immune responses. TH cells play a major role in determining which epitopes become targets for the immune response and selection of effector mechanisms. The TH cells select and activate appropriate effector cells including B cells that produce antibody and modulate the actions of other effector cells, Tc cells, NK cells, macrophages, granulocytes and antibody dependent cytotoxic (K) cells. TH-1 cells secrete IL-2 and IFN-γ which tend to activate macrophages and cytotoxic cells. TH-2 cells secrete IL-4, IL-5, IL-6 and IL-10 and tend to increase production of eosinophils and mast cells and to enhance production of antibody including IgE and decrease the function of cytotoxic cells. It is therefore contemplated that under certain circumstances it may be desirable to administer one or more of the lymphokines secreted by TH-1 cells in order to mimic a TH-1-type response. On the other hand, it may be desirable to administer the lymphokines secreted by TH-2 cells in order to mimic a TH-2-type response.

It is also within the scope of the invention to stimulate the lymphocytes with one or more antigens, prior to, or at the same time as, lymphocytes are stimulated with an antibody to a lymphocyte surface receptor. Any of a number of different antigens can be used including tumoral, microbiological, etc.

Typically, the antitumor activity of the total lymphocyte population stimulated *in vitro* with anti-CD3 is increased *in vivo* upon the administration of IL-2 by about 20%, preferably by about 50%, more preferably by about 60%, and most preferably by about 80%. Alternatively stated, the anti-CD3 stimulated lymphocytes display enhanced immunotherapeutic activity, e.g., cytotoxicity or lymphokine production, *in vivo* as represented by a decrease in the tumor burden by at least about 20%, preferably by at least about 40%, more

preferably by at least about 60%, and most preferably by at least about 80%. For example, in a typical protocol untreated animals will usually develop greater than about 250 tumors, while animals treated with anti-CD3 stimulated cells from a total lymphocyte population and IL-2 will usually develop less than about 40 tumors.

Similar activity can result from cells stimulated *in vitro* with anti-CD3 for less than about 4 hours, and even for as short as 30 minutes. The proliferation and antitumor activity of these cells can occur not only in mice, but also in humans.

To decrease the tumor volume in a mammal having tumors, the immunotherapeutic activity of a lymphocyte population first is enhanced in accordance with any of the methods described above. The stimulated total lymphocyte population, depleted lymphocyte population or stimulated lymphocyte subset then is administered to a mammal having tumors. Optionally, the mammal is pretreated with an immunosuppressant that preferably also is chemotherapeutic. While not intending to be bound by any theory, the use of an immunosuppressant may serve to suppress the activity of other immune cells thereby permitting the stimulated lymphocytes to function more effectively upon administration to the mammal or the immunosuppressant may serve to diminish the total volume of the tumor. Usually, immunosuppressants such as doxorubicin or cyclophosphamide (cytoxan) are used, although those skilled in the art readily recognize that other immunosuppressants can be used in accordance with the present invention such as corticosteroids.

The tumors which have shown a reduction upon treatment with cells stimulated by the method of the present invention and IL-2 include MCA-38 (colon carcinoma), RENCA (renal cell carcinoma), MCA-102 (sarcoma) and 38C (lymphoma) which are all tumors having different embryonic origins. The colon carcinoma is derived from cells which embryologically were derived from the ectoderm (the origin of the lining of all organs

as well as the skin), the renal cell carcinoma is derived from cells which embryologically were derived from the neural crest (origin of nervous tissue, gonads and adrenal glands), and the sarcoma is derived from cells which embryologically were derived from the connective tissue (bone, ligaments). Therefore, it is expected that tumors derived from cells that embryologically are derived from ectoderm, endoderm and mesoderm will respond to therapy.

T lymphocytes can also function as suppressor cells, i.e., they have regulatory mechanisms by which they can stop an on-going immune response. Therefore, it is expected that some of the T lymphocytes activated during the preparation of T-AK cells will be useful in the treatment of diseases where the immune response produces damage to normal tissues, e.g., autoimmune diseases such as Lupus erythematosus, multiple sclerosis, rheumatological conditions (rheumatoid arthritis), allergic conditions, and the rejection of transplanted organs such as kidney, liver, heart, lung, or bone marrow (autologous or allogeneic).

The immunotherapeutic activity or cytotoxicity of the lymphocytes can be enhanced by separating at least one cell subset, or subpopulation, from the lymphocytes to form a depleted lymphocyte preparation wherein the separated cell subset down-regulates the immunotherapeutic activity or cytotoxicity of the lymphocytes. Preferably, this method reduces or eliminates a regulatory mechanism from the lymphocytes, which allows the remaining cells to more fully express their immune function. The immunotherapeutic activity of the remaining lymphocytes, as represented by a measure of the cytotoxicity or antitumor activity of the cells, can be increased by a factor of at least 1.2, preferably by a factor of at least about 2.0.

The depleted lymphocytes are preferably cultured in a first medium with an antibody to a lymphocyte surface

receptor, optionally in the presence of low doses of IL-2 (<300 units/ml) for less than about 24 hours.

Preferably, this method involves the depletion of T lymphocyte populations, such as PBL populations, before the initiation of culture with an antibody to a lymphocyte surface receptor. More preferably, the separated cells are $CD4^+$ or $CD8^+$ lymphocytes, or more specific subsets of each of these populations. The cytotoxicity, or antitumor activity can be specific or nonspecific lytic activity. Preferably, it is nonspecific lytic or LAK activity. As a result of the removal or depletion of specific cell subsets that inhibit antitumor activity, the remaining lymphocytes develop increased immunotherapeutic activity.

The lymphocytes, and more preferably peripheral blood mononuclear lymphocytes, can be depleted of specific T cell subsets by any method. Preferably, the PBLs are depleted of specific subsets by negative depletion using magnetic beads, immunoaffinity columns, or antibody coated polystyrene plates. Typically, this involves the labelling of the PBLs with an antibody to a lymphocyte surface receptor for the T cells that are to be removed from the total PBL population. This mixture of labelled and unlabelled cells are then mixed with goat anti-mouse IgG-coated magnetic beads. A complex of the beads and the labelled T cells, i.e., those cells complexed with the surface receptor antibody, is formed. The beads/labelled T cell complexes are then separated from the mixture using a magnetic separator. In this way, a specific T cell subset, or portion thereof, can be removed from the PBL mixture.

The specific immune cell subset removed can be any that down-regulates the immunotherapeutic activity, preferably the cytotoxic activity, of the total immune cell population. This can include: $CD4^+$, or any of its subsets such as 2H4 or 4B4; $CD8^+$, or any of its subsets; NK cells, or any of its subsets; macrophages; B cells; and the like. Preferably, the immune cell subsets

removed are T cell subsets, and more preferably they are $CD4^+$ or $CD8^+$ cells.

In general, a typical sample of PBLs from a sample of human whole blood contains about 20-30% $CD8^+$ cells and about 30-50% $CD4^+$ cells. In order to increase the immunotherapeutic activity, e.g, antitumor activity, of an immune cell population according to the present invention, the cells that inhibit or down-regulate the immunotherapeutic activity of the population need only be removed until an increase in the immunotherapeutic activity, as represented *in vitro* by cytotoxicity, by a factor of about 1.2 is observed in the remaining cell population. Preferably, in order to increase the immunotherapeutic activity of immune cells according to the present invention, the number of $CD4^+$ or $CD8^+$ cells are reduced in the depleted immune cell populations by at least about 75%, more preferably by at least about 90%.

Most preferably, however, a "substantially completely depleted" immune cell population, e.g., PBL population, contains less than about 5% of the cell subset removed. For example, a "substantially completely $CD4^+$-depleted immune cell population" contains less than about 5% $CD4^+$ cells. Thus, the method of the present invention includes separating at least about 50% preferably at least about 75%, and more preferably at least about 90% of the $CD4^+$ or $CD8^+$ cells from PBLs to increase the immunotherapeutic activity, e.g., antitumor activity, of the remaining "depleted" cell population.

Furthermore, a method has been developed to enhance the cytotoxicity of immune cells by first culturing an immune cell population to form a cultured immune cell population; separating or positively selecting a cell subset or subpopulation, that is capable of developing immunotherapeutic activity or cytotoxicity; and separately culturing or subculturing the selected cells in a second medium in the presence of a monoclonal antibody to a lymphocyte cell surface antigen, optionally in the presence of low doses of IL-2.

Preferably, by using this method and positively selecting a cell subpopulation, the immunotherapeutic activity, as represented *in vitro* by the cytotoxicity or cytokine production, of this subpopulation can be increased by a factor of at least about 1.2, preferably by a factor of at least about 2.0. Preferably, the separated cells are $CD4^+$ or $CD8^+$ lymphocytes, or subsets of each of these populations.

Specific T lymphocyte subsets or subpopulations can be selected or separated from an unseparated or total lymphocyte population, preferably PBLs, by any method. Preferably, the specific cell subpopulations are separated from the total populations by positive selection using fluorescence-labelled monoclonal antibodies. Typically, this involves adding a fluorescein isothiocyanate-conjugated MoAb or a phycoerythrin-conjugated MoAb to a cultured lymphocyte population, incubating the cells with the conjugate for 30 minutes at 4°C, washing the cells, and sorting or selecting out the labelled cells using a fluorescence-activated cell sorter. For positively selecting $CD8^+$ cells the monoclonal antibody OKT8 can be used, and for positively selecting $CD4^+$ cells the monoclonal antibody OKT4 can be used, both of which are available from the Ortho Division of Johnson & Johnson.

According to specific preferred embodiments of the invention, the culturing process involves stimulating the depleted lymphocytes with an antibody to a lymphocyte surface receptor for less than about 24 hours, optionally in the presence of IL-2. The antibody to a lymphocyte surface receptor is preferably an anti-CD3 MoAb which is an antibody against the antigen receptor complex CD3, such as OKT3. The antibody to a lymphocyte surface receptor can be any of a variety of monoclonal antibodies against a surface antigen receptor complex having mitogenic potential including, but not limited to, anti-CD2, anti-CD4, anti-CD28 or anti-CD11b. The stimulated depleted lymphocytes are infused into the tumor-bearing

mammal, and an effective amount of IL-2 is administered to the mammal.

The interleukin-2 is a commercially available T cell growth factor. It can be a naturally occurring IL-2, such as might be derived from cultured rat splenocytes, or it can be recombinant IL-2. It is believed that other lymphokines can also be used in the present invention either in low doses together with anti-CD3 *in vitro* or administered to the patient following infusion of anti-CD3 stimulated lymphocytes. These include IL-1, IL-4, IL-6, IL-12, interferon, etc. It is envisioned that they can be used alone, in sequence, or in combination with IL-2 as an adjunct to primary anti-CD3 stimulation.

Although free IL-2 can be used in the method of the present invention, the IL-2 is preferably incorporated into liposomes as a delivery system. These phospholipid vesicles can contain varying amounts of IL-2, or other cytokines or bioactive compounds, depending on the type of interaction between the solute and the phospholipid.

Many methods have been proposed for the preparation of liposomes. Most of these methods involve a form of aqueous hydration of the lipid, which may be either in a powdered form or as a dried film. One of the most widely used techniques is known as the film method. Briefly, lipids of the desired composition in solution with an organic solvent are dried in the form of a thin film on the walls of a round-bottomed flask. A bioactive compound can be included in the film at this stage. The dry film is hydrated by adding a suitable aqueous phase and gently swirling the flask. With a hydrophilic bioactive compound, an aqueous solution is used for hydration. The liposomes formed by this procedure generally have a number of concentric bilayers and are called multilammelar vesicles (MLVs).

The liposomal IL-2 used in the present invention is preferably prepared using dimyristyl phosphatidyl choline (DMPC, Avanti Polar Lipids, Pelham, AL). Liposomes are formulated utilizing a freeze/thaw and bath sonication

technique, according to the method described by P.M. Anderson et al., Cancer Research, 50, 1853-1856 (1990).

Several routes of administration can be used for the administration of liposomes, for example, intravenous, intratumoral, subcutaneous, intraperitoneal, and oral delivery. An important advantage of liposomal delivery is the change in tissue distribution and binding properties as compared to the free forms of the bioactive ingredient, resulting in enhanced therapeutic index and decreased toxicity.

The stimulated lymphocytes, made by any of the methods outlined above, are added to the mammal with liposomal IL-2. More advantageously, the stimulated lymphocytes are administered intravenously whereas the liposomal IL-2 is administered intraperitoneally. Usually, liposomal IL-2 is added periodically after the initial administration of stimulated lymphocytes. In addition, the liposomal IL-2 and the stimulated lymphocytes can be administered to the mammal a second time after the initial treatment. Preferably, the second treatment takes place about 1 week after the initial treatment, although a second treatment any time after about 4 days is effective in substantially eradicating the tumor. As used herein, the phrase "substantial eradication of tumor" denotes a reduction in tumor volume to a point where the tumor either is completely destroyed or is so small that it is not readily recognizable by conventional methods.

Mammals undergoing bone marrow engraftment have a compromised bone marrow cell population for a period of about 15 to about 25 days. Throughout this description, the term "compromised bone marrow cell population" denotes an incomplete or depleted bone marrow cell population when compared to the mammal's normal bone marrow cell population. For example, a compromised bone marrow cell population can include a bone marrow cell population that is insufficient to achieve homeostasis in the mammal's immune system. The present invention

therefore advantageously further provides a method of stimulating the proliferation of bone marrow cells to generate bone marrow cells more readily in a compromised bone marrow cell population. In addition, the stimulated lymphocytes provide an anti-tumor effect against residual tumor cells in the bone marrow upon introduction into the patient, for example, by means of intravenous infusion.

In accordance with this method, the immunotherapeutic activity of an immune cell population is enhanced in accordance with one of the methods described above. Preferably, the immunotherapeutic activity is enhanced by culturing positively selected $CD4^+$ cells in the presence of anti-CD3 for a period of less than about 24 hours. The resulting stimulated $CD4^+$ cells are capable of stimulating the proliferation of bone marrow cells as defined, for example, by an increase in the number of colony forming units.

Alternatively, methods of stimulating the production of bone marrow cells can be carried out in accordance with the present invention. First, bone marrow cells can be extracted and purified from a mammal using techniques well known in the art, and then incubated with the stimulated lymphocytes for a period of time sufficient to generate more bone marrow cells. Preferably, the cells are incubated for a period of about 10 to about 20 days and most preferably, about 14 days at about 37°C. In addition, cytokines such as GM-CSF, IL-3, KL and EPO can be added to the bone marrow during culturing. The bone marrow cells produced in accordance with this method then can be infused into a mammal having a compromised bone marrow cell population using known techniques, in particular, intravenous injection. The bone marrow cells may also be injected at the same time as the stimulated lymphocytes during a bone marrow transplant such that the stimulated lymphocytes stimulate the proliferation of additional bone marrow cells.

Alternatively, the stimulated lymphocytes, made by any of the methods described above, can be administered

to a mammal having a compromised bone marrow population using known techniques, *e.g.*, intravenous injection. Optionally, the mammal is pretreated with an immunosuppressant such as cytoxan, that preferably also is chemotherapeutic. Advantageously, the stimulated depleted immune cell population or the stimulated immune cell subset is administered periodically to the mammal, for example every four or five days. More advantageously, the stimulated depleted immune cell population or stimulated immune cell subset is administered with an additional cytokine such as IL-2, IL-3, GM-CSF, KL, EPO and the like. The administration of the stimulated lymphocytes serves to stimulate the proliferation of additional bone marrow cells in a compromised bone marrow cell population of the treated mammal to generate bone marrow cells more rapidly when compared to proliferation of an unstimulated compromised bone marrow cell population. In addition, the stimulated lymphocytes provide an anti-tumor effect against residual tumor cells in the bone marrow.

The following examples are set forth as representative of specific and preferred embodiments of the present invention. These examples are not to be construed as limiting the scope of the invention in any manner. It should be understood that many variations and modifications can be made while remaining within the spirit and scope of the invention.

Example 1

Tumor Preparation and Tumor Cell Lines

The murine tumor cell line, MCA-38, a weakly immunogenic murine colon adenocarcinoma, was induced by the subcutaneous (s.c.) injection of dimethylhydrazine in C57BL.6 mice according to the method of T.H. Corbett et al., Cancer Research, 35, 2434-2439 (1975), which is incorporated herein by reference. A MCA-38 tumor cell line was established in tissue culture media (TCM) with 10% fetal calf serum (FCS, available from GIBCO Laboratories, Grand Island, NY) and used as a target for

cytotoxicity assays. The MCA-38 was maintained by subcutaneous (s.c.) passage in C57BL.6 mice. The TCM consisted of Rosewell Park Memorial Institute (RPMI) 1640 medium (obtained from GIBCO), supplemented with 25 mM HEPES [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (available from GIBCO Laboratories), 2 mM L-glutamine (available from GIBCO Laboratories), and 100 units/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO). The cell line was maintained in tissue culture media.

Example 2

Preparation of IL-2 in Liposomes

Liposomes containing IL-2 for use in all Examples were prepared as follows. IL-2 (specific activity 1.5 x 10^7 units/mg Hoffmann-LaRoche, Inc., Nutley, NJ) containing 25 mg human serum albumin per 1 x 10^6 units IL-2 was diluted in Hanks' Balanced Salt Solution (HBSS, obtained from GIBCO Laboratories, Grand Island, NY, also available from SIGMA Chemical Co., St. Louis, MO) and added to dimyristyl phosphatidyl choline (DMPC, Avanti Polar Lipids, Pelham, AL). Liposomes were formulated utilizing a freeze/thaw and bath sonication technique, according to the method described by P.M. Anderson et al., Cancer Research, 50, 1853-1856 (1990), which is incorporated herein by reference. To standardize the quantity of lipid injected with each concentration of IL-2 tested, IL-2 liposomes were synthesized utilizing a constant ratio of lipid:IL-2 solution of 15 mg DMPC:0.2 ml IL-2 solution. Mice treated with IL-2 liposomes received 5.0 x 10^4 units IL-2 liposomes.

Example 3

Cells Stimulated with Anti-CD3 *In Vitro* and Their Effect on Hepatic Tumors *In Vivo*

Peripheral blood lymphocytes, or splenocytes, from C57BL.6 mice were obtained by extracting the spleen and crushing it with a glass stopper in a petri dish with HBSS. A single suspension was created by repeated pipetting. The splenocytes were purified over Ficoll-

Hypaque, (Pharmacia, Piscataway, NJ), washed twice in phosphate buffered saline (PBS, pH 7.4 available from GIBCO, Grand Island, NY) and placed in culture in RPMI 1640 with 5% fetal calf serum, and 2% by volume of anti-CD3 supernatant 145-2C11 at a concentration of 2 x 10^6 cells/ml without IL-2 (anti-murine CD3 MoAb 145-2C11, which has been identified by O. Leo et al., Proc. Natl. Acad. Sci. USA, 84, 1374-1378 (1978), is available from ATCC). Cells were harvested the next morning, washed 3 times in HBSS, and resuspended in HBSS prior to intravenous injection. The concentration of anti-CD3 in the supernatant of 145-2C11 hybridoma is approximately 1-2 micrograms/ml.

For comparison purposes, splenocytes were stimulated with anti-CD3 (145-2C11) and IL-2 (100-300 units/ml) in the following manner. Cells were separated by Ficoll-Hypaque, washed 3 times in PBS, and placed at 1-2 x 10^6 cells/ml in RPMI 1640 + 5% fetal calf serum + 2% by volume anti-CD3 (145-2C11). The cells were diluted every 48-72 hours such that the concentration of cells was maintained at approximately 1 x 10^6 cells/ml. The cells injected into mice were harvested on day 3 and 5 of culture.

Approximately 20 million cells treated either with the anti-CD3 MoAb alone, or with the anti-CD3 MoAb and IL-2, were injected intravenously (i.v.) into C57BL.6 mice bearing hepatic tumors of MCA-38 colon adenocarcinoma. Both groups of mice received IL-2 in liposomes (50,000 units/day) intraperitoneal (i.p.) for five days. The number of hepatic tumors were determined on day 14 by double blind counting of surface tumors. The results are listed below in Table 1. These results demonstrate that cells stimulated with anti-CD3 for only 18 hours *in vitro* will develop antitumor activity *in vivo* in the presence of IL-2. The antitumor activity is comparable to that of cells cultured *in vitro* in anti-CD3 + IL-2 for 3-5 days.

Table 1

Effect of Adoptive Cells and IL-2 Liposomes on Number of Hepatic Tumors

Mouse	Anti-CD3 Alone for 18 hrs.	Anti-CD3 + IL-2 for 3-5 days
1	72	89
2	59	72
3	61	68
4	57	51
5	43	47
6	37	33
7	10	24
8	5	15
9	0	6
Mean	38.2	45
Median	43	47
Sd	26.9	27.9
Se[1]	8.9	9.3

[1] Both groups show equivalent antitumor activity *in vivo*.

Example 4

Cells Stimulated with Anti-CD3 *In Vitro* and Their Effect on Hepatic Tumors *In Vivo*

Lymphocytes obtained from murine spleens, as described above in Example 3, were stimulated with the anti-CD3 monoclonal antibody 145-2C11 overnight, i.e., for approximately 12-18 hours as described above in Example 3.

Approximately 15 million of these cells were injected intravenously (i.v.) into C57BL.6 mice bearing hepatic tumors of MCA-38 colon adenocarcinoma. The mice also received IL-2 in liposomes (50,000 units/day) intraperitoneal (i.p.) for five days. The number of hepatic tumors were determined on day 14 as described above in Example 3.

The controls were C57BL.6 mice injected with tumor cells in a similar manner as the treated mice; however, these mice were treated with IL-2 liposomes (50,000

units/day) only. The results are listed below in Table 2. These results demonstrate that with as little as 15 million cells stimulated for 24 hours with anti-CD3, there is a significant reduction in the number of hepatic tumors.

Table 2

Effect of 15 Million Adoptive Cells and IL-2 Liposomes on Number of Hepatic Tumors

Mouse	Controls	Anti-CD3
1	246	51
2	231	62
3	241	34
4	211	48
5	234	21
6	251	31
7	235	26
8	219	38
9	232	27
10	201	19
Mean	230.1	35.7
Sd	15.6	14.0
Se	4.9	4.4
p (unpaired student T Test)		0.001

Example 5

Titration of CD3 Stimulated Cells Intravenously

Splenocytes, obtained as described above in Example 3, were stimulated with OKT3 and IL-2 as described above in Example 3.

Nine week old C57BL.6 female mice (Harlan) bearing hepatic tumors of MCA-38 colon adenocarcinoma were divided into seven groups. The mice in the three groups intravenously received 5 million, 10 million, or 20 million cells stimulated with anti-CD3 and cultured in the presence of 100 units/ml IL-2 on days 3 and 5. All

mice, including a control group, which did not receive adoptively transferred cells, received 50,000 units IL-2 liposomes intraperitoneal qd., i.e. everyday, on days 3-7. For the mice in each of the groups that received adoptively transferred cells, the number of hepatic tumors were determined on day 14 as described above in Example 3. The results are presented below in Table 3. These results demonstrate that there is a distinct correlation between the quantity of cells administered and the number of tumors the mice develop. The mice receiving more cells developed fewer tumors.

This finding is important because a controlling factor in the infusion of cells is the culturing time. Cells cultured for a long period of time increase in size and produce respiratory complications and even death when infused. The stimulation of the cells for less than 24 hours, however, does not induce changes in size, making the cells virtually nontoxic during the administration.

Table 3

Titration of CD3 Stimulated Cells Intravenously

Mouse	Controls	5 Million	10 Million	20 Million
1	300	182	94	20
2	300	300	88	17
3	302	300	107	0
4	300	169	74	7
5	300	75	62	38
6	300	155	153	21
7	300		123	60
8			94	38
9			74	
Mean	300	196.8	96.6	25.1
Median	300	175.5	94.0	20.5
Sd	0.756	88.2	28.0	19.3
Se	0.286	36.0	9.3	6.8

Example 6

Preparation of T-AK Cells for T Cell Infiltration Experiment

C57BL.6 murine splenocytes were harvested and activated with anti-murine CD3 MoAb 145-2C11 and interleukin-2 (IL-2,) as described by P.M. Anderson et al., J. Immunol., 142, 1383-1394 (1989), which is incorporated herein by reference. The IL-2 used had a specific activity of 1.5×10^7 units/mg, and is available from Hoffmann-LaRoche. Fresh splenocytes were incubated in culture flasks at a concentration of 1.0×10^6 cells/ml of tissue culture media (TCM). The TCM consisted of RPMI-1640, supplemented with 25 mM HEPES [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 2 mM L-glutamine, 5% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mM nonessential amino acids (Gibco), 100 mM sodium pyruvate (Gibco, Grand

Island, NY), and 25 μM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO).

To generate T-activated killer cells, i.e., T-AK cells, 2 μg of 145-2C11 MoAb was added per one ml of TCM. In addition, IL-2 was added to the TCM at a concentration of 100 units/ml. Anti-CD3 MoAb was added only at the start of the culturing process. All cultures were supplemented with fresh TCM containing IL-2 to produce a cell density of 0.2-0.5 x 10^6 cells/ml every two to three days. Cultures were sampled on days 3, 5, 7, and 9; cell viability and increases in cell number were determined by the commonly used trypan blue exclusion procedure. Cells utilized in the adoptively transferred T cell infiltration experiment were from day 5, 7, and 9 of culture.

Example 7

Adoptively Transferred T Cells Infiltrate MCA-38 Hepatic Tumors

The ability of adoptively transferred T cells with cytolytic activity (T-AK cells) to infiltrate MCA-38 hepatic tumors was evaluated using congenic mice. T-AK cells were generated from splenocytes of C57BL.6 mice that express Thy 1.2 on their lymphocytes, as described in Example 8. Mice of the congenic strain B6:PL-Thy-1^a/Cy (available from Frederick Cancer Research Center and Development) that express Thy 1.1 on their T cells were inoculated with MCA-38 and then treated with T-AK cells from Thy1.2 mice (C57BL.6), IL-2 liposomes prepared as described above in Example 2, or a combination of IL-2 liposomes and T-AK cells.

Liver tissue was collected every 48 hours beginning the day after therapy was started (day 4 post tumor inoculation), and evaluated for the number of Thy 1.2^+ cells, Lyt.2.2^+ cells, L3T4^+ cells, and Mac 1^+ cells infiltrating the hepatic tumors. Ethidium bromide was added to facilitate identification of the early micrometastases within the liver, according to the method of J.L. Platt et al. described in J. Exp. Med., 157, 17

(1982). Tetraethylrhodamine isothiocyanate conjugated rabbit antiserum to human basement membrane (TRIC) was used to allow differentiation of intravascular cells from infiltrating cells, according to the method of J.L. Platt et al. described in J. Exp. Med., 158, 1227 (1983).

Monoclonal antibodies were purified from ascites utilizing saturated ammonium sulfate, washed twice with 50% ammonium sulfate, dialyzed against PBS, and adjusted to a final concentration of 500 μg/ml in PBS. The antibody recognizing the cell marker Lyt.2 is 2.43 (ATCC Cat. No. TIB 210), and that recognizing the cell marker L3T4 is GK1.5 (ATCC Cat. No. TIB 207). Rat anti-Thy 1.2 FITC was obtained from Becton Dickinson (Mountain View, CA) and anti-MAC-1 from Boehringer Mannheim (Indianapolis, IN).

Mice treated with IL-2 liposomes alone demonstrated host Lyt.2$^+$ cells infiltrating the hepatic tumors within 24 hours of i.p. injection. The number of infiltrating lymphocytes increased over the subsequent 48 hours and remained constant thereafter. A much smaller number of Mac 1$^+$ cells (including both PMNs and monocytes) also infiltrated the tumors of these mice. Rare L3T4$^+$ cells were also noticed. T-AK cells (Thy1.2$^+$) infused without IL-2 liposomes into Thy 1.1$^+$ mice were observed in the tumors two days after initial injection. Rare Mac 1$^+$ cells were also observed, however, no L3T4$^+$ cells were observed.

When T-AK cells were administered with IL-2 liposomes, large numbers of Lyt.2$^+$ cells were noted in the tumors. Of these, approximately 60% Thy 1.2$^+$ represented adoptively transferred cells, while 40% were of host origin. These cells were apparent 48 hours after therapy began. A moderate number of Mac 1. cells were seen but only rare L3T4$^+$ cells were found. The cellular infiltrate was present up to day 11 of protocol (3 days after the end of therapy) when the mice were sacrificed.

Example 8

In Vivo Growth of Anti-CD3 Activated Cells

The data presented in Table 4 demonstrate that the cells activated with anti-CD3 overnight (T-activated killer cells; T-AK) will proliferate *in vivo*. C57BL.6 mice were injected intravenously with 15 x 10^6 anti-CD3 activated cells obtained from B6:PL Thy-1^a/Cy mice. These two groups differ in only one genetic characteristic, i.e. they are congenic strains, and therefore, do not reject each other's tissues. However, this genetic characteristic allows the determination of the origin of the lymphocytes proliferating *in vivo*. C57BL.6 mice express the LY5.2 allele, while the B6:PL Thy-1^a mice express the Ly5.1 allele.

Splenocytes from LY5.1 (B6:PL Thy-1^a) mice were activated as previously described. Briefly, purified splenocytes were cultured in tissue culture media (TMC) containing 3% volume:volume of the supernatant from the 145-2C11 hybridoma (Hamster anti-mouse anti-CD3 MoAb) for a period of 18 hours. The activated cells were then injected intravenously into LY5.2 (C57BL.6) mice. These mice also received intraperitoneal (i.p.) IL-2-liposomes (OncoTherapeutics Inc.) at a dose of 50,000 units once a day i.p. for 5 days. Mice were sacrificed on days 2 and 10 after injection at which time the following parameters were measured:

1. Percent $CD4^+$ and $CD8^+$ lymphocytes.
2. Percent T cells expressing the IL-2 receptor (TAC^+ as a sign of activation.)
3. The percentage of LY5.1 cells in the spleen.
4. The number of white blood cells in the peripheral blood and spleen.

On day 0 (after stimulation of LY5.1 cells with anti-CD3, but prior to infusion) the T cells (CD4 and CD8) in the stimulated culture expressed an increased level of IL-2 receptor (78 and 92%) (Table 4). This is an increase from baseline which usually is ≤10% in a normal unstimulated animal. As observed, the percentage

of $CD8^+$ cells increased *in vivo* over time while the $CD4^+$ cells decreased. The number of LY5.1 cells in the spleen of the LY5.2 recipient animal increased over time until day 10 where 34% of the spleen cells were of .LY5.1 origin, confirming their expansion *in vivo*. In addition, the number of white blood cells in the peripheral blood increased dramatically to 88,000/mm^3. The data confirm the *in vivo* proliferation of cells activated *in vitro* with anti-CD3.

Table 4

In Vivo Growth of Anti-CD3 Stimulated Splenocytes

(Donor Ly 5.1^+, Recipient Ly 5.2^+)

Day After Transfer	% Positive		%Ly 5.1 in Spleen	Peripheral Blood WBC X 1000
	CD4 (TAC^+)	CD8 (TAC^+)		
0	23(78)	18(92)	-	2
2	17(21)	53(38)	15	3
10	11(3)	63(3)	34	68

Example 9

Regression of Tumor Size After Treatment With Anti-CD3 Stimulated Cells and IL-2 Liposomes

Murine lymphocytes stimulated overnight induce a significant reduction in the volume of subcutaneous tumors in mice bearing the MCA-38 colon carcinoma (Table 5). 500,000 MCA-38 tumor cells were injected subcutaneously into mice. After 10-15 days a nodule could be palpated at the site. These mice were injected with anti-CD3 stimulated murine splenocytes (using the same technique described in Table 4) and 50,000 U/day of i.p. IL-2 liposomes. Tumor measurements were performed every other day. The data presented in Table 5 demonstrate that only one out of ten mice receiving IL-2-liposomes with T-AK cells had tumor progressively grow while nine out of ten had complete tumor regression. All the mice in the groups receiving no therapy and 50% of

the mice receiving IL-2 liposomes + cytoxan developed tumors which progressively increased in size.

Table 5

	Day	28	31	35	41
Group	Tumor Volume in mm^3				
Ia-Control (HRSS)					
	1	++			
	2	7260	11492	++	
	3	8750	6800	10656	++
	4	7986	++		
	5	1296	8064	5800	15558
++ Mice sacrificed due to tumor growth					
IIa- Cytoxan + IL-2 liposomes					
	1	172	787	3971	7488
	2	0	0	0	6
	3	144	550	847	3726
	4	0	0	0	0
	5	0	0	0	0
	6	40	108	405	2916
	7	126	256	550	2916
	8	600	2746	3324	9802
	9	0	0	0	0
	10	0	0	0	0
IIIa- Cytoxan + IL-2 liposomes + T-AK cells					
	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
	6	0	0	0	0
	7	144	600	2156	7142
	8	0	0	0	0
	9	0	0	0	0
	10	0	0	0	0
TUMOR-MCA-38 (SQ) DOSE OF CELLS: 10 M (1 dose) IL-2-LIPOSOMES: 50 K/D X 5D					

Example 10

Increase in IL-2 Receptor Expression After Stimulation With Anti-CD3

IL-2 receptor (p55,CD25) is upregulated on human T lymphocytes after a 12 hour incubation with anti-CD3 (OKT3-Ortho) (Figure 1). Lymphocytes were cultured as described above and the expression of the IL-2 receptor was measured by flow cytometry using a fluorescein-labeled anti-CD25 MoAb against the p55 or β chain of the IL-2 receptor (Becton-Dickinson, CA). Human peripheral blood lymphocytes stimulated with doses as low as 1 ng/ml of OKT3 exhibited increased expression of the β chain of the IL-2 receptor. Also, incubation with anti-CD3 for periods of time as short as 1 hour followed by 12 hours in TCM alone, also resulted in the increased expression of CD25.

Human T lymphocytes also increase their expression of the α chain (p75) of the IL-2 receptor after overnight incubation with anti-CD3 (Figure 2). It is noted that the receptor itself has a high affinity for IL-2, as does the cell because it has so many receptors on it. The increased expression of both the α and β chains makes the IL-2 receptor expressed by these T cells a receptor with very high affinity of IL-2.

Example 11

In Vitro Proliferation of Human Lymphocytes Incubated with Anti-CD3

Human lymphocytes stimulated overnight with anti-CD3 proliferate when placed in media containing IL-2 (Figure 3). Human lymphocytes at a concentration of 3×10^6 cells/ml were incubated with 10 ng/ml of OKT3 at 4°C. Cells were washed twice with HBSS and were placed in tissue culture media (TMC) composed of RPMI 1640 or AIM-V supplemented with 25mM HEPES, 2mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 5% heat inactivated human AB serum (GIBCO) and 30, 100, 300, or 1000 units/ml of IL-2. Cells were counted on the days indicated and were diluted back to 0.5×10^6 cells/ml in

fresh TCM. As observed, by day 10 the number of cells had increased between 20 and 25 fold.

Example 12

OKT3 Induced Cell Proliferation

Lymphocytes stimulated with anti-CD3 for as short as 4 hours will proliferate adequately in the presence of IL-2. Lymphocytes were cultured with no OKT3, or 3 or 10 ng/ml of OKT3 for 4 hours. Lymphocytes were washed twice with HBSS and then were cultured in tissue culture media containing 100 u/ml of IL-2. As shown in Figure 4, either concentration of OKT3 tested induced cell proliferation.

Example 13

In Vivo Proliferation of Human T Cells Treated With anti-CD3 *In Vitro* and IL-2 *In Vivo*

Human T lymphocytes proliferate *in vivo* in cancer patients after receiving T-AK cells and IL-2. Patient lymphocytes were obtained by leucophaeresis one day prior to the initiation of treatment (day-1). The cells were purified over Ficoll-Hypaque and cultured overnight at a concentration of 3 X 10^6 cells/ml in AIM-V media with 10 ng/ml of OKT3. After overnight culture, the cells were washed 3 times in HBSS, resuspended in reinfusion medium (0.9% saline solution (Abott Laboratories) containing 25% human serum albumin (American Red Cross) and 30 U/ml of IL-2) and given back to the patients by intravenous infusion over 30 minutes. The patients then received IL-2 intravenously (3 million U/m2 continuous infusion and 1.5 million U/m2 bolus for a total of 7 days (proliferation has also been observed with lower doses of IL-2 being administered). Samples of peripheral blood were obtained from the patient on the days present in Figure 5 and a WBC count and differential counts were done.

There was a marked increase in the WBC after 6-8 days in therapy. This increase was on the average 4-6 fold over the baseline number of WBC. The WBC ranged up to 86,000 mm^3 in one patient. Only one patient out of 25

thus far has not shown any increase in peripheral WBC. This increase was preferential for the T cells ($CD3^+$), although other cells including the NK cells ($CD16^+/CD56^+$) increased in absolute number (Table 6). The $CD3^+$ T lymphocytes also had an increased expression of activation markers (Table 7) such as IL-2 receptor (CD25), HLA-Dr and CD38, demonstrating the presence of a large number of activated T cells in the circulation. Because of the similarity to the results obtained in the murine experiments, it is most likely that the cells proliferating in the peripheral blood are those which were stimulated with OKT3 *in vitro*.

Table 6

Cohort 2 - Surface Phenotype

Relative Date	% Positive			
	CD3	CD4	CD8	CD56
Baseline	76	49	28	10
Day 1 Culture	75	53	30	7
Day 7-9	83	39	43	16
Day 28	88	56	31	9
Day 29 Culture	84	57	31	6
Day 35-37	82	50	32	16

Table 7

Cohort 2 - Surface Phenotype

Relative Date	% $CD3^+$ Cells			
	HLA-DR	CD25	CD38	CD69
Baseline	4	6	31	3
Day 1 Culture	11	12	33	51
Day 7-9	55	31	82	7
Day 28	8	18	25	3
Day 29 Culture	17	32	26	50
Day 35-37	41	42	64	6

Example 14

Lymphocytic Infiltration of Tumors

$CD8^+$ cells infiltrate the site of tumor. In two patients in which tumor biopsies were obtained, a significant lymphocytic infiltration was observed as compared with the pre-treatment biopsy. Further analysis using well-established, routine techniques of immunohistology revealed that these cells were mostly $CD8^+$ cells.

Example 15

Treatment of Cancer Patients with Short-term Stimulated Depleted Lymphocytes

T-cells are purified from peripheral blood, lymph nodes, or any other source that provides T-cells, using methods well known to the skilled artisan. For example, peripheral blood lymphocytes (PBLs) are isolated from heparinized venous blood (human whole blood) by centrifugation over Ficoll-Hypaque according to the method of A. Boyum, Scand. J. of Clin. Lab. Invest., 99, 77 (1968), which is incorporated herein by reference. Isolated mononuclear cells are washed three times with phosphate buffered saline (PBS, pH 7.4) (GIBCO Laboratories, Grand Island, NY) and counted. $CD4^+$ and $CD8^+$ enriched cultures are obtained by negative depletion using magnetic beads (obtained from Baxter Healthcare

Corporation, Deerfield, IL; also available from Advanced Magnetics, Massachusetts; or Dynal Corp., Norway).

PBLs are labelled by incubation with either of the monoclonal antibodies OKT4 or OKT8 (Ortho, Raritan, NJ) for 30 minutes on ice. The cells are then washed twice with cold PBS and mixed with goat anti-mouse IgG-coated magnetic beads (obtained from Baxter Healthcare; also available from Dynal Corp., Norway) at a bead:cell ratio of 10:1. The bead/cell mixture is incubated for 30 minutes at 4°C while rotating at 5-6 rpm. At the end of the incubation, the bead/cell suspension was diluted two-fold with cold PBS. Using a magnetic separator (Baxter Healthcare Corporation, Deerfield, IL), the beads are allowed to collect against the side of the test tube for five minutes. The supernatant containing unbound cells is then collected and transferred to a new tube. This process is repeated three times to completely remove the beads and bead-bound cells. The cells that remain in suspension ($CD4^+$-depleted or $CD8^+$-depleted PBLs) are washed and counted.

The depleted lymphocyte population (5×10^6 cells) are cultured in 25 cm^2 flasks (Corning, Corning, NY) in 10 ml tissue culture medium (TCM). The TCM consists of Rosewell Park Memorial Institute (RPMI) 1640 medium (available from GIBCO, Grand Island, NY) supplemented with 25 mM Hepes [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (penicillin/streptomycin mix available from GIBCO, Grand Island, NY), and 5% pooled heat-inactivated human serum. The cultures are supplemented with 10 ng/ml of the anti-CD3 MoAb OKT3 (Ortho Division, Johnson & Johnson, Raritan, NJ). The cultures are optionally supplemented with low doses of IL-2, for example <300 units/ml of highly purified recombinant human IL-2 from E. coli (Hoffman-LaRoche, Nutley, NJ). (See, A. Wang et al., Science, 224, 1431 (1984); and S.A. Rosenberg et al.,

Science, 223, 1412 (1984), which are incorporated herein by reference.)

Cultures are incubated at 37°C in a humidified atmosphere of 5% CO_2 for less than about 24 hours, preferably 12 to 18 hours, and for as little as 4 hours or 30 minutes. The lymphocytes are incubated in roller bottles, hollow fibers, gas permeable bags, or regular tissue culture flask. The lymphocytes are washed with PBS and placed in a suspension of saline solution, human serum and IL-2 (75 U.ml). The stimulated and depleted T lymphocyte preparation is administered to the patient, preferably with IL-2, most preferably with IL-2 liposomes.

Example 16

Treatment of Cancer Patients with a Short-term Stimulated Positively-Selected T Lymphocyte Subset

T-cells are purified from a known source, such as from PBLs, as described above. The cells are resuspended in tissue culture medium and plated onto polystyrene plates previously coated with anti-CD4 or anti-CD8. The lymphocytes are incubated at 4°C for 45 to 60 min. The unbound cells are washed off by rinsing the plates with PBS. The bound cells are enriched for $CD4^+$ or $CD8^+$ lymphocytes.

The positively selected lymphocyte subset (5×10^6 cells) is cultured in RPMI 1640 medium supplemented with 25 mM Hepes [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (penicillin/streptomycin mix available from GIBCO, Grand Island, NY), and 5% pooled heat-inactivated human serum. The cultures are supplemented with 10 ng/ml of the anti-CD3 MoAb OKT3 (Ortho Division, Johnson & Johnson, Raritan, NJ). The cultures are optionally supplemented with low doses of IL-2, for example <300 units/ml of highly purified recombinant human IL-2 from *E. coli* (Hoffman-LaRoche, Nutley, NJ). (See, A. Wang et al.,

Science, 224, 1431 (1984); and S.A. Rosenberg et al., Science, 223, 1412 (1984), supra.)

Cultures are incubated at 37°C in a humidified atmosphere of 5% CO_2 for less than about 24 hours, preferably 12 to 18 hours, and for as little as 4 hours or 30 minutes. The lymphocyte subset is incubated in roller bottles, hollow fibers, gas permeable bags, or regular tissue culture flask. The lymphocyte subset is washed with PBS and placed in a suspension of saline solution, human serum and IL-2 (75 U.ml). The stimulated lymphocyte subset is administered to the patient, preferably with IL-2, most preferably with IL-2 liposomes.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention. The relevant portions of the references cited herein are incorporated by reference.

What is Claimed is:

1. A method of treating a tumor-bearing mammal, comprising the steps of:

(a) stimulating lymphocytes *in vitro* in the presence of an antibody to a lymphocyte surface receptor for less than about 24 hours, optionally in the presence of interleukin-2 (IL-2), to form stimulated lymphocytes;

(b) infusing the stimulated lymphocytes into said mammal without substantial further *in vitro* culture; and

(c) administering to said mammal an amount of a lymphokine, effective to induce proliferation of said stimulated lymphocytes and to enhance the immunotherapeutic activity of the lymphocytes *in vivo*, resulting in a decrease in the tumor burden by at least about 20%;

wherein said lymphocytes are a subset of T lymphocytes.

2. The method of claim 1, wherein said antibody to a lymphocyte surface receptor is selected from the group consisting of anti-CD3, anti-CD2, anti-CD4, anti-CD5, anti-CD28 and anti-CD11b.

3. The method of claim 1, wherein said lymphokine is selected from the group consisting of IL-1, IL-2, IL-4, IL-6, IL-12 and interferon.

4. The method of claim 1, wherein said subset is a $CD4^+$ subset.

5. The method of claim 1, wherein said subset is a $CD8^+$ subset.

6. The method of claim 1, wherein the step of stimulating in the presence of anti-CD3 is carried out for about 12-18 hours.

7. The method of claim 1, wherein step (a) includes stimulating in the presence of a low dose of IL-2 for about 12-18 hours.

8. The method of claim 1, wherein in step (c), the IL-2 is administered encapsulated in liposomes.

9. The method of claim 1, wherein said tumor burden is decreased by at least about 40%.

10. The method of claim 1, wherein said tumor burden is decreased by at least about 60%.

11. The method of claim 1, wherein the tumor-bearing mammal is a human.

12. The method of claim 11, wherein said lymphokine is IL-2 and is administered in about 3 million U/m2 continuous infusion and 1.5 million U/m^2 bolus per day.

13. The method of claim 11, wherein said lymphokine is IL-2 which is administered at a dose of about $1x10^7$ to $1.8x10^7$ IU/m^2/day.

14. The method of claim 1, wherein prior to the step of stimulating lymphocytes *in vitro* in the presence of an antibody to a lymphocyte surface receptor, said lymphocytes are contacted with an anti-CD8 antibody to produce $CD8^+$-depleted lymphocytes.

15. The method of claim 1, wherein prior to the step of stimulating lymphocytes *in vitro* in the presence of an antibody to a lymphocyte surface receptor, said lymphocytes are contacted with an anti-CD4 antibody to produce $CD4^+$-depleted lymphocytes.

16. A method of enhancing the immunotherapeutic activity of immune cells comprising the steps of:

(a) separating at least one cell subpopulation from a T lymphocyte population to form a depleted T lymphocyte population; wherein the cell subpopulation removed is capable of down-regulating the immunotherapeutic activity of the T lymphocyte population; and

(b) stimulating the depleted T lymphocyte population in the presence of an antibody to a lymphocyte surface receptor for less than about 24 hours, optionally in the presence of IL-2, to provide a stimulated depleted T lymphocyte population, wherein the stimulated depleted T lymphocyte population exhibits increased immunotherapeutic activity compared to a similarly treated undepleted T lymphocyte population.

17. The method of claim 16, wherein said antibody to a lymphocyte surface receptor is selected from the

group consisting of anti-CD3, anti-CD2, anti-CD4, anti-CD5, anti-CD28 and anti-CD11b.

18. The method of claim 16, wherein the step of separating cells comprises separating $CD4^+$ lymphocytes from the T lymphocyte population to form a $CD4^+$-depleted T lymphocyte population.

19. The stimulated $CD4^+$-depleted T lymphocyte population produced by the method of claim 18.

20. The method of claim 16, wherein the step of separating cells comprises separating $CD8^+$ lymphocytes from the T lymphocyte population to form a $CD8^+$-depleted T lymphocyte population.

21. The stimulated $CD8^+$-depleted T lymphocyte population produced by the method of claim 20.

22. A method of enhancing the immunotherapeutic activity of immune cells comprising the steps of:

(a) positively selecting at least one T lymphocyte subset from a T lymphocyte population; and

(b) stimulating said T lymphocyte subset in the presence of an antibody to a lymphocyte surface receptor for less than about 24 hours, optionally in the presence of IL-2, to provide a stimulated T lymphocyte subset, wherein the stimulated T lymphocyte subset exhibits increased immunotherapeutic activity compared to a similarly treated T lymphocyte population.

23. The method of claim 22, wherein said antibody to a lymphocyte surface receptor is selected from the group consisting of anti-CD3, anti-CD2, anti-CD4, anti-CD5, anti-CD28 and anti-CD11b.

24. The method of claim 22, wherein the step of selecting a T lymphocyte subset comprises selecting $CD4^+$ lymphocytes from the T lymphocyte population to form a $CD4^+$ T lymphocyte subset.

25. The stimulated $CD4^+$ T lymphocyte subset produced by the method of claim 24.

26. The method of claim 22, wherein the step of selecting T lymphocytes comprises selecting $CD8^+$

lymphocytes from the T lymphocyte population to form a $CD8^+$ T lymphocyte subset.

27. The stimulated $CD8^+$ T lymphocyte subset produced by the method of claim 26.

28. A method of stimulating the proliferation of bone marrow cells comprising the steps of:

(a) enhancing the immunotherapeutic activity of an immune cell population according to the method of claim 15 or 21; and

(b) incubating the immune cell population of step (a) with bone marrow and IL-2.

29. A method of enhancing the engraftment of bone marrow cells in a patient comprising the steps of:

(a) enhancing the immunotherapeutic activity of an immune cell population according to the method of claim 15 or 21; and

(b) administering said immune cell population of step (a) to a bone marrow transplant patient.

Upregulation of IL2 Receptor After 24 hours of OKT3 Stimulation

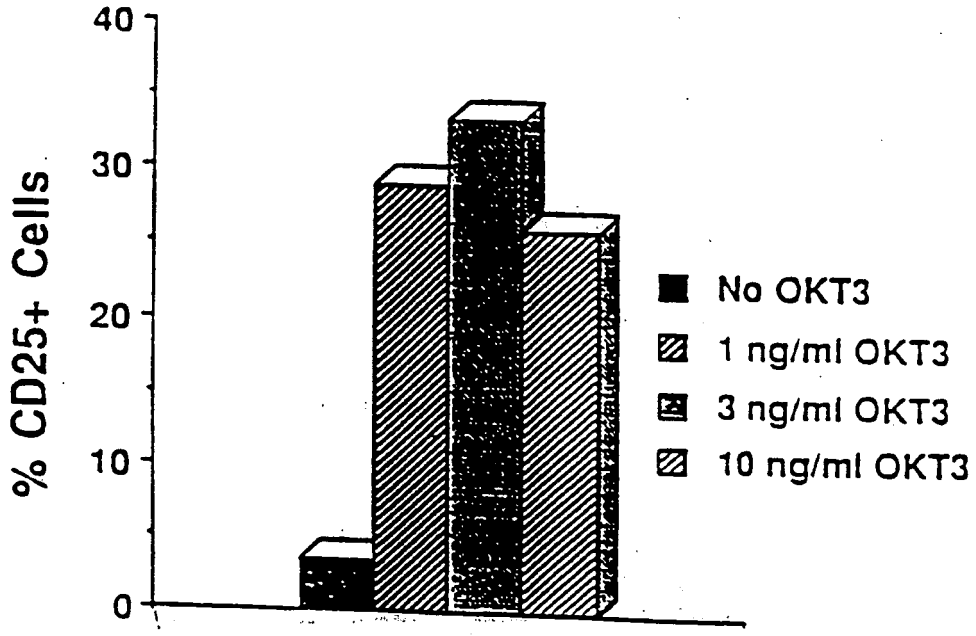

FIGURE 1

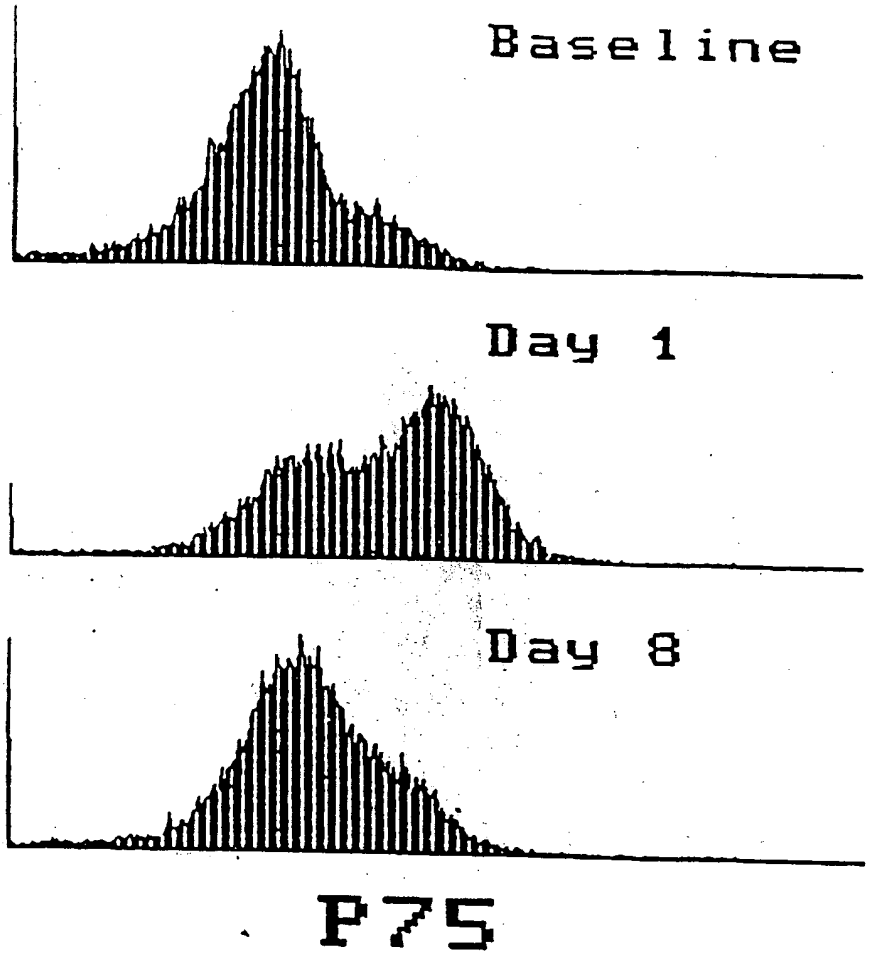

FIGURE 2

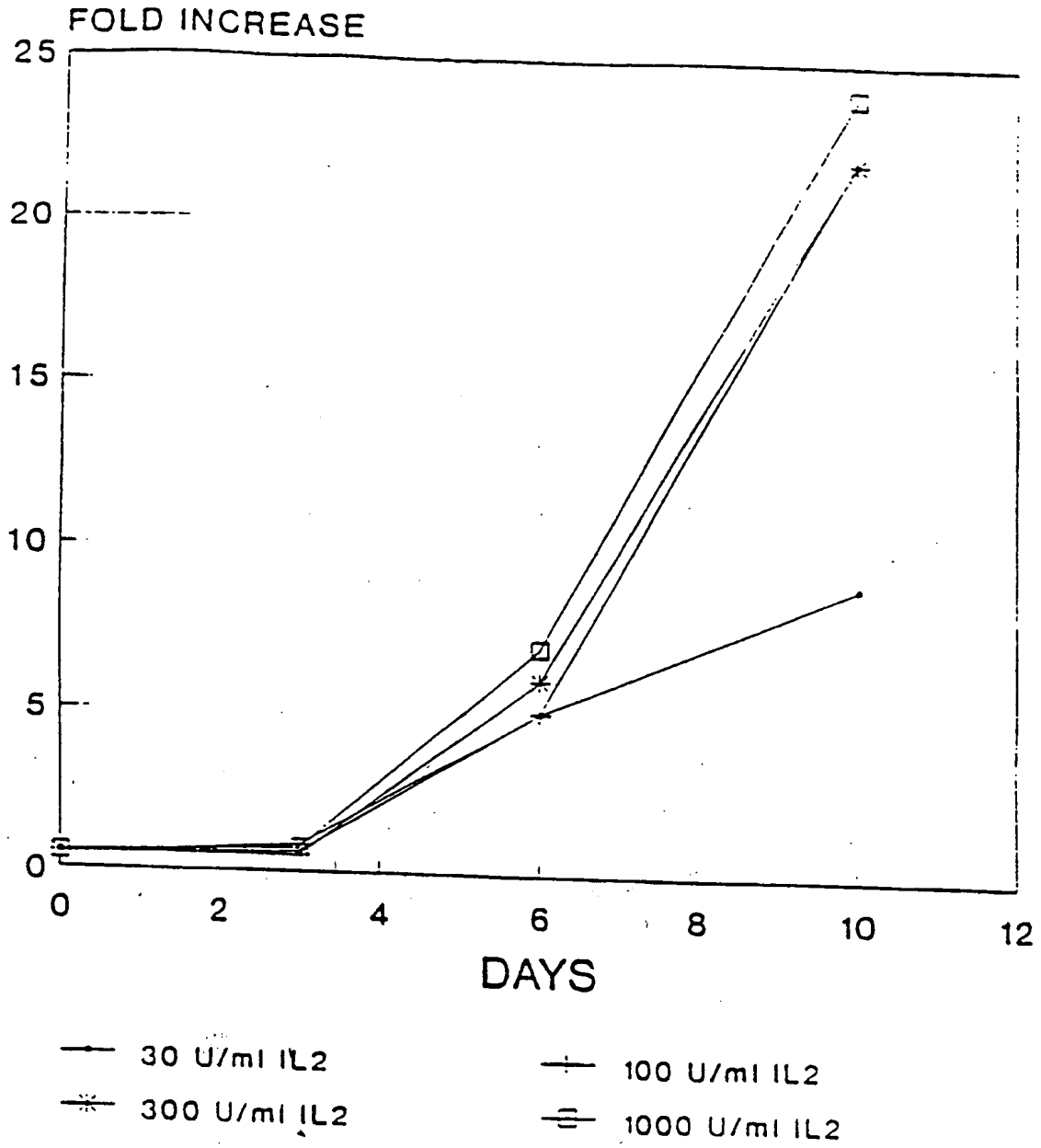
FOLD INCREASE
25
20
15
10
5
0
0
2
4
6
8
10
12
DAYS
30 U/ml IL2
100 U/ml IL2
300 U/ml IL2
1000 U/ml IL2

FIGURE 3

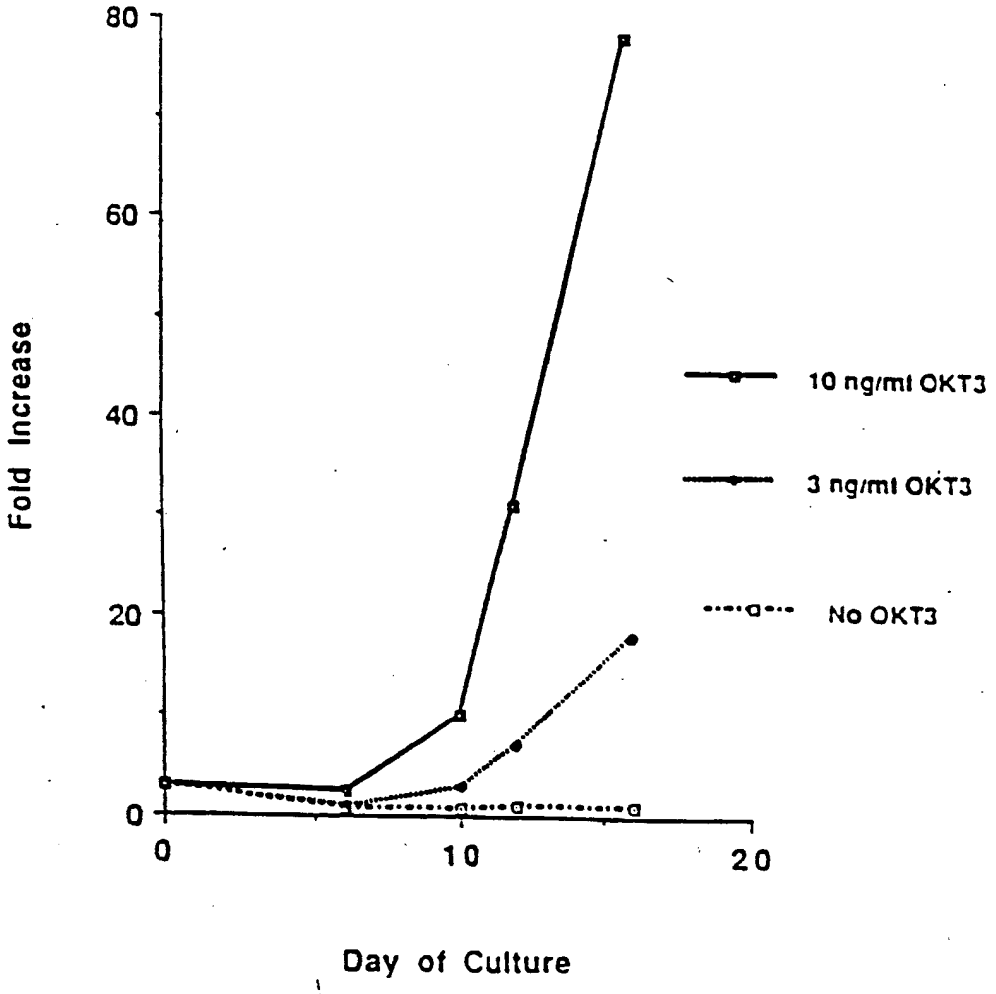
80
60
40
20
0
Fold Increase
0
10
20
Day of Culture
10 ng/ml OKT3
3 ng/ml OKT3
No OKT3

FIGURE 4

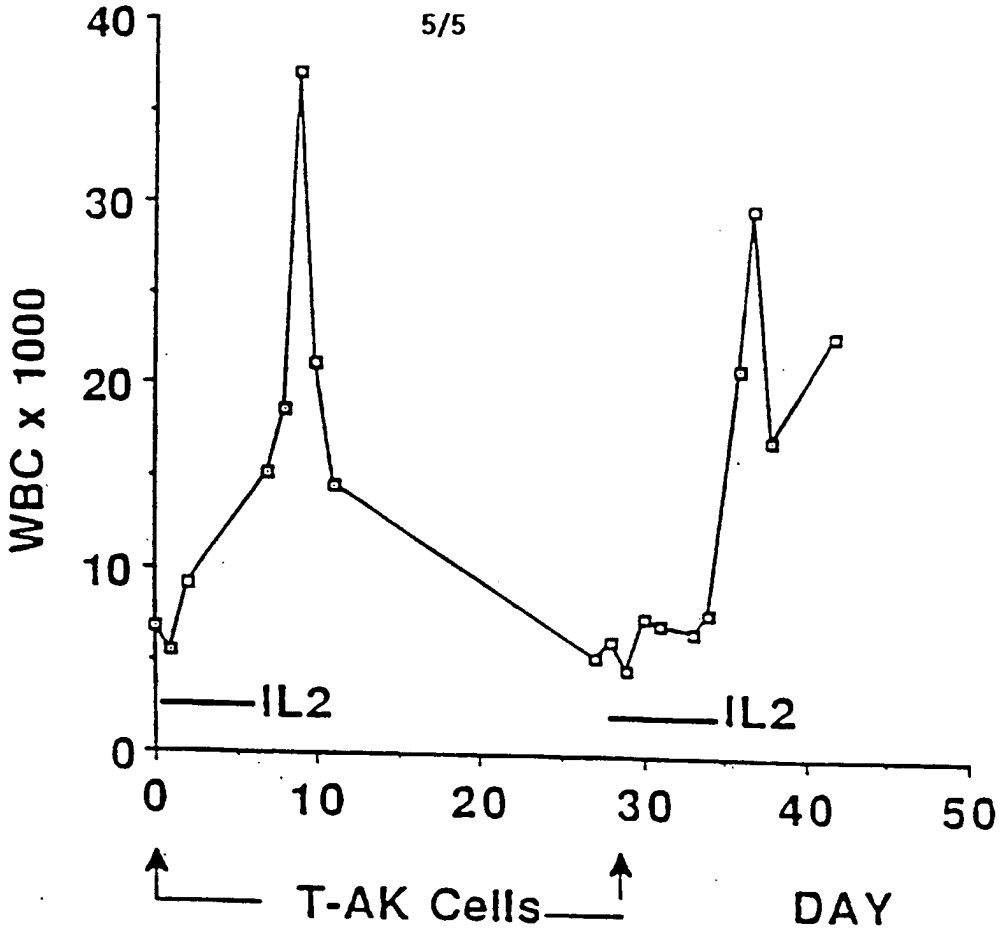

FIGURE 5

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/US95/06703

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/21, 45/00; C12N 5/08; C07K 14/52, 14/55, 14/705, 16/28
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.1, 93.71, 93.7; 435/240.2, 240.21; 530/351, 388.22, 388.73, 388.75, 389.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, DERWENT WPI, EMBASE, CHEM AB search terms: author names, lak, til, cd3, okt3, cd4, cd8, okt4, okt8, bone marrow, antibody

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 93/00918 (OCHOA ET AL.) 21 JANUARY 1993, SEE ENTIRE DOCUMENT.	1-29
Y	EP, A, 0,409,655 (SEKINE) 23 JANUARY 1991, SEE ENTIRE DOCUMENT.	1-29
Y	JOURNAL OF IMMUNOLOGY, VOLUME 146, ISSUED 15 MAY 1991, R.L. GELLER, "GENERATION OF LYMPHOKINE-ACTIVATED KILLER ACTIVITY IN T CELLS", PAGES 3280-3288, SEE ENTIRE DOCUMENT.	1-29

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 JULY 1995	21 AUG 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RON SCHWADRON Telephone No. (703) 308 0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06703

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/85.1, 93.71, 93.7; 435/240.2, 240.21; 530/351, 388.22, 388.73, 388.75, 389.6